Studies on the Phosphorylation of the Inhibitory Subunit of Troponin during Modification of Contraction in Perfused Rat Heart

By PAUL J. ENGLAND

Department of Biochemistry, Medical School, University of Bristol,
Bristol BS8 1TD, U.K.

(Received 20 May 1976)

1. Rat hearts were perfused with $^{32}\text{P}_i$, and contractile force was increased by positive inotropic agents (agents that increase contractility). The inhibitory subunit of troponin (troponin I) was then isolated by affinity chromatography in 8M-urea, and its ^{32}P content measured. Incorporation of phosphate into the subunit was calculated on the basis of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ specific radioactivity in the hearts. 2. When hearts were perfused with 30nM-DL-isoprenaline (*N*-isopropylnoradrenaline), there was an increase in contractile force over 30s which was paralleled by an increase in troponin I phosphorylation. When hearts were perfused for 25s with increasing concentrations of isoprenaline from 1 nM to 0.6 μM , there was again a parallel increase in contractile force and troponin I phosphorylation. The maximum phosphorylation observed was 1.5 mol of phosphate/mol of troponin I, which was reached after 25s with 0.1 μM -isoprenaline. 3. Hearts were stimulated with a 15s pulse perfusion of 30nM-DL-isoprenaline. There was an increase in contractile force which was followed by a return to the control value within 50s. Troponin I phosphorylation increased to a plateau value which was reached within 30s, and remained constant for 60s after the isoprenaline pulse. Phosphorylase *a* and 3':5'-cyclic AMP concentration showed changes similar to that of the contractile force. There was no change in 3':5'-cyclic GMP concentration. 4. When hearts stimulated with a 15s pulse of isoprenaline were subsequently perfused with 0.6 μM -acetylcholine, the changes in contractile force, phosphorylase *a* and 3':5'-cyclic AMP were very similar to those seen with the 15s pulse of isoprenaline alone. Troponin I phosphorylation increased to a maximum 30s after the end of the isoprenaline pulse, but then rapidly decreased during the subsequent 30s. This decrease was preceded by a 60% increase in the concentration of 3':5'-cyclic GMP. 5. Hearts were perfused with 0.2 μM -glucagon for periods up to 60s. Contractile force showed little change for the first 30s, but then increased rapidly. This was paralleled by changes in 3':5'-cyclic AMP concentration. Troponin I phosphorylation increased slowly, but the increase in contractile force had reached a maximum before significant phosphorylation had occurred. 6. It is concluded that under certain conditions, e.g. immediately after β -adrenergic stimulation, there is a good correlation between contractile force and troponin I phosphorylation. However, under other conditions, e.g. when contractile force is decreasing after removal of β -adrenergic stimulation or in the presence of glucagon, contractile force and troponin I phosphorylation are not well correlated. These results suggest that mechanisms for modifying cardiac contractility, other than troponin I phosphorylation, must be present in rat heart.

The increase in contractility which occurs in heart muscle in response to adrenergic stimulation is now thought to be mediated via changes in the concentration of 3':5'-cyclic AMP (Sutherland *et al.*, 1968; Sobel & Mayer, 1973; Kukovetz *et al.*, 1975; Osnes & Øye, 1975). However, the intracellular mode of action of 3':5'-cyclic AMP in this response is not understood. Two hypotheses involving modification of proteins by phosphorylation have been proposed. One involves the phosphorylation of sarcoplasmic reticulum or sarcolemma (reviewed in Katz *et al.*, 1975; Krause *et al.*, 1975), leading to changes in intracellular

Ca^{2+} distribution and subsequent changes in contractility. The second hypothesis involves phosphorylation of contractile proteins, in particular troponin (Stull *et al.*, 1972; England *et al.*, 1973). Troponin is composed of three subunits, a Ca^{2+} -binding subunit (troponin C), an inhibitory subunit (troponin I) and a tropomyosin-binding subunit (troponin T). Both troponin I and troponin T have been shown to be phosphorylated *in vitro* by phosphorylase kinase and 3':5'-cyclic AMP-dependent protein kinase in skeletal muscle (Bailey & Villar-Palasi, 1971; Stull *et al.*, 1972; England *et al.*, 1973;

Perry & Cole, 1973) and cardiac muscle (Reddy *et al.*, 1973; Cole & Perry, 1975; Rubio *et al.*, 1975). Cole & Perry (1975) have shown, however, that with whole troponin as substrate, the highest rate of phosphorylation was with cardiac troponin and cardiac 3':5'-cyclic AMP-dependent protein kinase. Under these conditions only troponin I was phosphorylated, and this suggests that troponin phosphorylation is likely to be of more physiological significance in cardiac muscle.

The hypothesis as proposed by Stull *et al.* (1972) and England *et al.* (1973) suggested that phosphorylation of troponin could modify its properties to allow an increased interaction of the contractile elements. This could be attained either by a decrease in the inhibitory properties of troponin, or by changing its Ca^{2+} sensitivity. The finding of Rubio *et al.* (1975) that phosphorylation of troponin in guinea-pig cardiac native actomyosin caused a lowering of the Ca^{2+} concentration required to activate actomyosin adenosine triphosphatase gave support to this hypothesis. Experiments showing that phosphorylation of troponin I occurred in intact perfused rat heart on stimulation with adrenaline (England, 1975) provided additional evidence. These results showed that there was a time-dependent increase in contractile force, and that these parallel increases in phosphorylation and contractile force resulted from stimulation at various concentrations of adrenaline. The present work extended the results obtained with adrenaline by using other agents that change the contractility of the perfused rat heart, and by measuring the state of phosphorylation of troponin I. By choosing a variety of conditions and performing detailed time-course analyses of the responses, it was hoped that these experiments would provide a rigorous test *in vivo* of the hypothesis that changes in troponin I phosphorylation are closely associated with changes in cardiac contractility.

Materials and Methods

Heart perfusions

These were carried out as previously described (England, 1975). Briefly, hearts from male Wistar rats (300–400 g) were removed under barbiturate-induced anaesthesia and perfused by the Langendorff technique at constant pressure (6 kPa) with bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 11 mM-glucose but with the P_i concentration lowered to 0.234 mM to increase the $^{32}\text{P}_i$ specific radioactivity (England & Walsh, 1976). After 5 min of pre-perfusion, to allow recovery after removal from the animal, the hearts were perfused with medium containing approx. $10 \mu\text{Ci}$ of $^{32}\text{P}_i/\text{ml}$ for 15 min in a recirculating system. The purpose of the perfusion with $^{32}\text{P}_i$ was to permit incorporation of ^{32}P into intracellular ATP (γ position) (England & Walsh, 1976), which was then assumed to be the phosphate donor to troponin I during phosphorylation. The

validity of this assumption is discussed in the Results and Discussion sections. After this the hearts were given a 1 min perfusion by drip-through with non-radioactive medium. Substances modifying the contractile response were then administered at constant concentrations by continuation of the drip-through perfusions. Contractile force at the peak of the length-tension curve was monitored with a force-displacement transducer (Devices Instruments Ltd., Welwyn Garden City, Herts., U.K.) attached by thread to the apex of the heart. For measurements of cyclic nucleotide concentrations, hearts were perfused as above but omitting $^{32}\text{P}_i$ from the medium. Contractile force and phosphorylase α were measured in these hearts to confirm the similarity of response to those perfused with $^{32}\text{P}_i$. At the end of the perfusions the hearts were rapidly frozen (Wollenberger *et al.*, 1960) and powdered under liquid N_2 .

Analytical methods

The concentration of ATP in neutralized HClO_4 extracts of hearts was measured as described by Lamprecht & Trautschold (1963) with hexokinase and glucose 6-phosphate dehydrogenase, and gave values of 16–22 $\mu\text{mol/g}$ of dry heart in control perfusions. In a small number of hearts (less than 5%) the ATP concentration was less than 16 $\mu\text{mol/g}$, and the results obtained with these hearts were ignored. [γ - ^{32}P]ATP specific radioactivity in heart extracts was measured by incorporation of ^{32}P into phosphorylase as previously described (England, 1975; England & Walsh, 1976).

The concentration of 3':5'-cyclic AMP and 3':5'-cyclic GMP were measured in heart extracts prepared by deproteinization with 0.6 M-trichloroacetic acid, followed by removal of the acid with water-saturated diethyl ether. 3':5'-Cyclic AMP was assayed by the protein-binding displacement assay of Brown *et al.* (1971). 3':5'-Cyclic GMP assays were kindly performed by Dr. Janet Albano, Department of Pharmacology, University of Bristol. This method used a specific antibody for 3':5'-cyclic GMP, separation of bound and free 3':5'-cyclic GMP being achieved by Millipore-membrane (0.45 μm pore size) filtration. The material that assayed as 3':5'-cyclic GMP in heart extracts could be destroyed on treatment with phosphodiesterase, and the amount assayed was linearly related to the amount of extract used.

The proportions of phosphorylase in α and β forms was measured essentially as described by Stull & Mayer (1971). A small amount (30–40 mg) of frozen heart powder was homogenized in 1 ml of 60% (v/v) glycerol/20 mM-NaF/1 mM-EDTA, pH 7.5, at -40°C . The extract was gradually allowed to thaw during homogenization, 1 ml of 20 mM- β -glycerol phosphate, pH 6.8, was then added, and the extract centrifuged for 5 min at 10000g. Phosphorylase activity in the

Fig. 1. Distribution of protein and radioactivity, after polyacrylamide-gel electrophoresis with sodium dodecyl sulphate, in troponin I purified by affinity chromatography

Troponin I was purified from rat heart perfused with $^{32}\text{P}_i$ by affinity chromatography as described in the text. Electrophoresis in 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate was carried out as described by Weber & Osborn (1969), with $20\mu\text{g}$ of troponin I per gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue, and the E_{600} measured by using a Gilford linear transport system attached to a spectrophotometer (a). The gel was subsequently sliced into 3 mm transverse sections which were dissolved in 30% (w/v) H_2O_2 and counted by liquid-scintillation spectrometry (b) as described by Tishler & Epstein (1968). The molecular weight of the protein was determined as described by Weber & Osborn (1969), using as standard proteins: phosphorylase *b*, pyruvate kinase, lactate dehydrogenase and cytochrome *c*.

presence and absence of 2mM-AMP in the supernatant was assayed as described by Hardman *et al.* (1965). The production of glucose 1-phosphate from glycogen in the presence of 50mM- P_i was measured by a coupled fluorimetric system with phosphoglucomutase and glucose 6-phosphate dehydrogenase. The proportion of phosphorylase *a* in the extracts was calculated as the ratio of activities without and with AMP divided by 0.7 (0.7 is the ratio of activities of pure phosphorylase *a* without and with AMP).

Troponin I was isolated, with a purity exceeding 96% as measured by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, by the affinity-chromatography method of Syska *et al.* (1974). Details of the method as applied to rat

heart extracts are given in England (1975). Fig. 1 shows a typical absorbance profile of purified troponin I after polyacrylamide-gel electrophoresis, and also the distribution of ^{32}P in the gel. There was essentially one protein band only, and all the radioactivity migrated with this band. The yield of troponin I was approx. 0.3 mg/g of wet heart, and was independent of the extent of phosphorylation. This indicated that the affinity chromatography was not purifying a particular phosphorylation state of troponin I.

During the purification of phosphorylated proteins, a major problem is to ensure that dephosphorylation or additional phosphorylation does not occur by enzymic or chemical action. The affinity-chromatography procedure for isolation of troponin I was carried out in the presence of 8M-urea, which was only removed at the final stage when the troponin I was pure. The possibility of enzymic modification of the phosphorylation state of troponin I during this procedure was therefore minimized. However, experiments were performed that directly tested the efficiency of 8M-urea in preventing protein modification. Cardiac troponin was isolated [by a modification of the method of Greaser & Gergely (1971)] and phosphorylated in troponin I by cardiac 3':5'-cyclic AMP-dependent protein kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Ray & England, 1976b). The ^{32}P -labelled troponin was then mixed with non-radioactive perfused heart powder, and troponin I was isolated by affinity chromatography. More than 85% of the added ^{32}P was recovered in the troponin I fraction, showing that little or no dephosphorylation had occurred during isolation. The small loss of radioactivity that occurred was probably caused by incomplete binding of troponin I to the affinity column, rather than dephosphorylation. In a second experiment, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to a non-radioactive perfused heart powder, and troponin I isolated. No radioactivity was associated with the troponin I, showing that no additional phosphorylation occurred during isolation. The ^{32}P present in troponin I was converted into mol of phosphate by using the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ specific radioactivity as previously described (England, 1975).

Materials

Phosphorylase *b* was prepared as described by Fischer & Krebs (1958), and phosphorylase kinase as described by Cohen (1973). 3':5'-Cyclic AMP-dependent protein kinase from ox heart muscle was prepared up to the DEAE-cellulose-column-chromatography step as described by Reimann *et al.* (1971) for the skeletal-muscle enzyme. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared by the method of Glynn & Chappell (1964). Radioactive isotopes were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and biochemicals from Boehringer Corp. (London)

Ltd., London W.5, U.K. or Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. DL-Isoprenaline (*N*-isopropylnoradrenaline) was from Kodak Research Chemicals, Kirkby, Liverpool, U.K. All other chemicals were from BDH Chemicals, Poole, Dorset, U.K. or Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Results and Discussion

Perfusions with isoprenaline

In a previous study (England, 1975), contractile force and troponin I phosphorylation were measured in perfused rat hearts stimulated with adrenaline. A maximum incorporation of approx. 1 mol of phosphate/mol of troponin I was obtained. Cole & Perry (1975), however, reported that 2 mol of phosphate/mol of troponin I could be incorporated into bovine cardiac troponin by incubation *in vitro* with 3':5'-cyclic AMP-dependent protein kinase. In view of the lower phosphorylation obtained in rat hearts perfused with adrenaline, an attempt was made to incorporate additional phosphate into troponin I in the perfused heart. Isoprenaline is a very potent β -adrenergic agonist (Goodman & Gilman, 1970),

and was used in the perfusion system in an attempt to increase troponin I phosphorylation above that obtained with adrenaline. In addition, isoprenaline has little α -adrenergic activity (Goodman & Gilman, 1970), and the use of this agent would also indicate whether the phosphorylation of troponin I was mediated solely via the β -adrenergic receptor.

Fig. 2 shows the time-course of changes in contractile force and troponin I phosphorylation in hearts perfused with 30 nM-DL-isoprenaline. After a short lag period contractile force increased to approx. 170% of the control value, reaching a plateau after 25–30 s. The control value for troponin I phosphorylation was initially low (approx. 0.2 mol of phosphate/mol of troponin I) but increased to a plateau value of 1.1 mol/mol over a time-course that was very similar to the increase in contractile force. This time-course, and the parallel changes in contractile force and phosphorylation, were very similar to those seen with adrenaline (England, 1975).

Fig. 3 shows a dose-response curve for hearts perfused with concentrations of DL-isoprenaline from 1 nM to 30 μ M. Hearts were perfused for 25 s with the agonist before being freeze-clamped. There was a linear increase in contractile force with increasing

Fig. 2. Time-course of increase in contractile force (●) and 32 P incorporation into troponin I (Δ) during perfusion of rat hearts with 30 nM-DL-isoprenaline

Hearts were perfused with 32 P_i as described in the Methods and Materials section, and then perfused by drip-through during a control period. The hearts were freeze-clamped at the times shown after continuous perfusion with 30 nM-DL-isoprenaline. Other details are given in the text. The increase in force of contraction is expressed as a percentage of that during the control period. Each point is the mean of results from three hearts, and the vertical bars are 2 s.e.m.

Fig. 3. Dose-response curve of increase in contractile force (●) and incorporation of 32 P into troponin I (Δ) at various concentrations of DL-isoprenaline

Hearts were perfused with 32 P_i as described in Fig. 2, and then perfused by drip-through for 25 s with the concentration of DL-isoprenaline shown. Each point is the mean of results from three hearts, and the vertical bars are 2 s.e.m.

Fig. 4. Correlation between increase in contractile force and incorporation of ^{32}P into troponin I in perfused rat hearts

The data were taken from Figs. 2 and 3 (■), where the hearts were perfused with DL-isoprenaline, or from England (1975) (○), where the hearts were perfused with adrenaline.

isoprenaline concentrations from 1 nM to 0.1 μM , but no further increase occurred at higher concentrations. The phosphorylation of troponin I also showed a similar response, although a certain amount of variability was seen. The maximum phosphorylation found was 1.5 mol of phosphate/mol of troponin I, which was considerably higher than that found with adrenaline stimulation (England, 1975). This result shows that more than one site on troponin I can be phosphorylated *in vivo*, as was found *in vitro* by Cole & Perry (1975). The significance of this additional phosphorylation is discussed below.

The phosphorylation of troponin I described above was estimated on the basis of ^{32}P incorporation, with the assumption that the phosphate donor was $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. If the troponin I contained non-radioactive phosphate, or if the phosphate donor had a specific radioactivity different from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the heart, the phosphorylation values of Figs. 2 and 3 would be erroneous. The troponin I within each group of hearts shown in Figs. 2 and 3 were combined, and total phosphate was determined (Itaya & Ui, 1966) after alkaline hydrolysis (Meisler & Langan, 1969) as previously described (England, 1975). In no case did the estimate of total phosphate differ by more than 20% from that calculated by ^{32}P incorporation.

This result agreed with that found in the series of hearts perfused with adrenaline (England, 1975), and showed the validity of using ^{32}P to measure total phosphate incorporation.

Fig. 4 is a replot of the data of Figs. 2 and 3, with the percentage increase in contractile force, plotted against troponin I phosphorylation. The data from the study with adrenaline (England, 1975) are also included for comparison. The data obtained by using isoprenaline as the positive inotropic agonist (i.e. the agent causing an increase in contractility) were very similar to those with adrenaline. This suggests that the same (or very similar) mechanism, i.e. interaction of the agonist with the cardiac β -adrenergic receptor, was mediating the response to both adrenaline and isoprenaline.

Fig. 4 shows that the plot of increase in contractile force against troponin I phosphorylation appeared biphasic. Where the amount of phosphate in troponin I was less than 1.0 mol/mol there was linear correlation between increase in contractile force and troponin I phosphorylation. Above this value, increased phosphorylation was not accompanied by increased contraction. The significance of this biphasic plot is not clear at present, but it may indicate similarities with other phosphorylated proteins in which more than one phosphorylation site is present. Cohen and co-workers have demonstrated that both phosphorylase kinase and glycogen synthetase can be phosphorylated at multiple sites (Cohen & Antoniwi, 1973; Nimmo & Cohen, 1974; Cohen *et al.*, 1975). With phosphorylase kinase, the enzyme activity was controlled by phosphorylation of one site, and the dephosphorylation of this site was controlled by the phosphorylation state of the other. Phosphorylation of the two sites in glycogen synthetase had synergistic effects on enzyme activity. It is possible that the phosphorylation of troponin I in perfused rat heart may show similar features to these enzyme systems. If it is assumed that phosphorylation of one site only in troponin I is necessary to change cardiac contractility, and that the phosphorylation of a second site has another (at present unknown) function, the curve of Fig. 4 can be explained. However, before this hypothesis can be accepted, it will be necessary to show that phosphorylation of the first site changes the properties of troponin in such a way as to be consistent with changes in cardiac contractility. In addition it must be shown that during the initial part of the curve of Fig. 4, only one site in troponin I is being phosphorylated, and that partial phosphorylation at two sites does not occur.

Pulse perfusions with isoprenaline

The data above, and that previously published (England, 1975), showed a good correlation between

Fig. 5. Time-course of changes in perfused rat heart after a 15s pulse of DL-isoprenaline

Hearts were perfused with $^{32}\text{P}_i$ as described in Fig. 2, and perfused by drip-through for 15s with 30nM-DL-isoprenaline. Perfusion was continued by drip-through for the time shown with control medium, when the hearts were freeze-clamped. Other perfusion details and assays are given in the Methods and Materials section. (a) ●, Percentage increase in contractile force over the control value; △, ^{32}P incorporated into troponin I; (b) ▲, phosphorylase *a* as a percentage of the total phosphorylase; ○, concentration of 3':5'-cyclic AMP; ■, concentration of 3':5'-cyclic GMP. Each point is the mean of results from at least three hearts, and the vertical bars are 2S.E.M.

troponin I phosphorylation and an increase in contraction during the period immediately after stimulation with the agonists, when increases in both contractile force and phosphorylation were occurring. In the experiments described in this section, an attempt was made to study the dephosphorylation of troponin I, after prior stimulation, when contractile force was decreasing. A 15s pulse of a submaximal dose (30nM) of isoprenaline caused a 60% increase in force within 30s of the introduction of the agonist, followed by a decrease to the control value after a further 40s (Fig. 5a). However, the phosphorylation of troponin I reached a maximum of 0.8mol of phosphate/mol of troponin I and remained elevated even after contractile force had decreased to the control value. These data showed a lack of correlation between troponin I phosphorylation and force of contraction, indicating that the state of phosphorylation of troponin I was not the only determinant of cardiac contraction, at least under conditions where it was decreasing.

Fig. 6. Time-course of changes in perfused rat heart after a 15s pulse of DL-isoprenaline followed by continuous perfusion with acetylcholine

Hearts were perfused as described in Fig. 5 with a 15s pulse of 30nM-DL-isoprenaline. After this the perfusion was continued by drip-through with medium containing 0.6 μM -acetylcholine, and the hearts were freeze-clamped at the times shown. (a) ●, Percentage increase in contractile force over the control value; △, ^{32}P incorporated into troponin I; (b) ▲, phosphorylase *a* as a percentage of the total phosphorylase; ○, concentration of 3':5'-cyclic AMP; ■, concentration of 3':5'-cyclic GMP. Each point is the mean of results from at least three hearts, and the vertical bars are 2S.E.M.

To investigate this result further, the concentrations of 3':5'-cyclic AMP and 3':5'-cyclic GMP were measured, and also the proportion of phosphorylase in the phosphorylated (*a*) form (Fig. 5b). 3':5'-Cyclic AMP concentration reached a peak rapidly, before the maximum response of the other measured variables. This suggested that increases in 3':5'-cyclic AMP could be an initial event in response to isoprenaline, and agreed with the hypothesis that increases in the contractility of the heart are mediated by changes in 3':5'-cyclic AMP (Sobel & Mayer, 1973). The proportion of phosphorylase in the active form increased rapidly, reached a peak value 25s after the start of the isoprenaline perfusion, and then decreased to a value significantly above the control value. This decrease occurred at the same time as troponin I phosphorylation was reaching a maximum, and suggested that the phosphatase catalysing the

dephosphorylation of phosphorylase *a* was not active towards phosphorylated troponin I. This result was noteworthy in that in skeletal muscle there appeared to be phosphatases with activity towards troponin I which did not remove phosphate from phosphorylase *a* (Ray & England, 1976a), and similar enzymes could also be present in heart.

In an attempt to cause dephosphorylation of troponin I, perfused hearts were first stimulated with a 15 s pulse of 30 nM-isoprenaline, followed by a continuous perfusion with 0.6 μ M-acetylcholine. Preliminary experiments indicated that this concentration of acetylcholine caused a 15–20% lowering of the rate of beating in control hearts, and this slowing was also seen in the hearts perfused with acetylcholine after a pulse of isoprenaline (Fig. 6). Fig. 6(a) shows that the response of the contractile force was the same as that in the absence of acetylcholine (Fig. 5a), but that after approx. 30 s with acetylcholine there was a dephosphorylation of troponin I. Even under these conditions, however, there was a decrease in contractile force which occurred before the dephosphorylation of troponin I. Fig. 6(b) shows that acetylcholine had little effect on the time-course or extent of the changes of 3':5'-cyclic AMP or phosphorylase *a* observed after a pulse of isoprenaline (cf. Fig. 5), but acetylcholine caused an elevation of the 3':5'-cyclic GMP concentration after approx. 20 s. This increase preceded by 10–20 s the dephosphorylation of troponin I. Similar effects of acetylcholine on heart rate, force of contraction and concentrations of 3':5'-cyclic GMP have been noted in unpaced hearts (George *et al.*, 1973). It is not possible to conclude from these data that the increase in 3':5'-cyclic GMP is the direct stimulus for troponin I dephosphorylation, but it has been reported that certain phosphoprotein phosphatases are inhibited by 3':5'-cyclic AMP via a phosphorylation mechanism (Huang & Glinsman, 1975). It is therefore possible that the dephosphorylation of troponin I in these experiments was caused by stimulation (by an unknown mechanism) of a phosphatase by 3':5'-cyclic GMP, but experiments with isolated phosphatase preparations will be necessary to substantiate this.

Interpretation of these experiments was partially complicated by the decrease in heart rate seen with acetylcholine, and it is possible that the changes in troponin I phosphorylation caused by this agent were secondary to this effect. To resolve this, it will be necessary to repeat these experiments under conditions where heart rate is kept constant by periodic electrical stimulation. However, George *et al.* (1973) showed that in rat heart changes in 3':5'-cyclic GMP and contractile force with acetylcholine were obtained whether or not the heart rate was kept constant. This complication did not arise during perfusions with positive inotropic agents, as little or no increase in heart rate occurred.

Perfusions with glucagon

Although the major physiological site of action of glucagon is probably the liver, it has been known for some time that glucagon will increase the contractility of cardiac muscle (Farah & Tuttle, 1960). This effect required greater concentrations of glucagon than normally found in the mammalian circulation. The increase in contraction was accompanied by increases in 3':5'-cyclic AMP and phosphorylase *a*, but these were not prevented by the β -adrenergic blocking agent propranolol (Lucchesi, 1968; Mayer *et al.*, 1970). These results were interpreted as indicating that glucagon was activating adenylate cyclase, but via a receptor different from that of catecholamines (Mayer *et al.*, 1970). The time-course of these changes with glucagon was also slower than that seen with adrenaline. Because of these differences, troponin I phosphorylation in perfused rat heart was investigated during stimulation with 0.2 μ M-glucagon (Fig. 7). There was a slight increase in contraction during the first 30 s, but a rapid increase then occurred to reach a value of 160% of the control after 40 s. The time-course of changes in 3':5'-cyclic AMP

Fig. 7. Time-course of changes in perfused rat heart during perfusion with 0.2 μ M-glucagon

Hearts were perfused with 32 P_i as described in Fig. 2, and were then perfused by drip-through with 0.2 μ M-glucagon for the time shown, when the hearts were freeze-clamped. Other details are given in the Methods and Materials section. (a) ●, Percentage increase in contractile force over the control value; Δ, 32 P incorporated into troponin I; (b) ▲, phosphorylase *a* as a percentage of total phosphorylase; ○, concentration of 3':5'-cyclic AMP. Each point is the mean of results from three hearts, and the vertical bars are 2S.E.M.

(Fig. 7b) was very similar, with a slight increase during the first 30 s with glucagon, followed by a rapid larger increase. The time-course of troponin I phosphorylation was quite different from this (Fig. 7a). There was a slow continuous rise in phosphorylation which was still increasing after 60 s, and the marked increase in contraction that occurred between 30 and 40 s was not accompanied by any comparable increase in phosphorylation. When hearts were stimulated with adrenaline or isoprenaline, increased contraction was always accompanied by increased troponin I phosphorylation. Fig. 4 shows that with either of these agonists an increase in contraction of 60% was accompanied by phosphorylation of troponin I to a value of 0.7–0.8 mol of phosphate/mol of troponin I. During the glucagon perfusions, however, a 60% increase in contraction occurred when the troponin I contained only 0.3–0.4 mol of phosphate/mol. The time-course of troponin I phosphorylation was very similar to that seen for phosphorylase interconversion (Fig. 7b). However, these data should not be interpreted as suggesting a role for phosphorylase kinase in troponin I phosphorylation in perfused rat heart. Although Stull *et al.* (1972) and England *et al.* (1973) reported a rapid phosphorylation of skeletal-muscle troponin I by phosphorylase kinase, work by Cole & Perry (1975) has shown that cardiac troponin is a much better substrate for 3':5'-cyclic AMP-dependent protein kinase.

General discussion

The initial purpose of the experiments described in this paper was to show whether increases in contractile force were always accompanied by increased troponin I phosphorylation in perfused rat heart, and whether the extent of phosphorylation was related to the force of contraction. The experiments showed that with adrenaline, isoprenaline and glucagon, troponin I phosphorylation was increased when contractile force was increased. However, study of the time-courses of these changes showed certain discrepancies. In particular, with glucagon perfusion the increase in contractile force preceded a significant increase in phosphorylation. Also, the experiments using pulse perfusions with isoprenaline showed that when contractile force was decreasing after previous stimulation, there was no corresponding fall in troponin I phosphorylation. There were thus two distinct situations in which troponin I phosphorylation was not correlated with contractile force. The hypothesis outlined in the introduction suggested that phosphorylation of troponin I caused an increase in Ca^{2+} sensitivity of the actomyosin system, and that this resulted in increased contractile force without the necessity for changes in intracellular Ca^{2+} (Stull *et al.*, 1972; England *et al.*, 1973). The results above showing that troponin I phosphorylation was not

always associated with increased contractile force indicate that this hypothesis is unable to explain completely the mode of action of agents that increase contractility.

It is unlikely, however, that the phosphorylation of troponin I has no role in modulation of contraction. The maximum rate of phosphorylation seen in the perfused rat heart was equivalent to 30 nmol of phosphate transferred/min per g wet wt. of heart. The total 3':5'-cyclic AMP-dependent protein kinase in rat heart is approx. 0.1 unit/g wet wt. (1 unit is equivalent to 1 μmol of phosphate transferred/min) with histone as substrate at 37°C (J. D. Corben & S. Keely, personal communication). Cardiac troponin I (as native tropomyosin) is phosphorylated at approx. 35% of the rate of histone (K. P. Ray & P. J. England, unpublished work). These calculations show that a large proportion of the total protein kinase activity will be required to phosphorylate troponin I during the initial response to β -adrenergic agonists (Figs. 2, 5 and 6, and England, 1975). This calculation assumes that 3':5'-cyclic AMP-dependent protein kinase is the enzyme actually phosphorylating troponin I *in vivo*, and that the extracted activity is the total present in the tissue. Although the results in perfused rat heart show that control of contraction cannot be exclusively mediated by troponin I phosphorylation, the fact that troponin I is a major phosphorylated protein in heart suggests a role for this effect. The observation of Rubio *et al.* (1975) that phosphorylation of troponin I in guinea-pig cardiac actomyosin increased the adenosine triphosphatase sensitivity to Ca^{2+} also supports this conclusion. However, results in this laboratory (Ray & England, 1976b) have indicated that phosphorylation of troponin I causes a decrease in Ca^{2+} sensitivity of the actomyosin adenosine triphosphatase from ox and rat cardiac muscle. The discrepancy between these observations and those of Rubio *et al.* (1975) is difficult to explain, although there could be species differences. These results are difficult to fit into the hypothesis for explaining increased cardiac contractility through troponin I phosphorylation. Taken in addition to the findings in perfused heart, they throw considerable doubt on a direct action of troponin I phosphorylation in mediating the changes in contractility.

As phosphorylation of troponin I cannot explain changes of contraction in heart, it is necessary to consider other mechanisms for the action of positive inotropic agents. 3':5'-Cyclic AMP has been firmly implicated in this effect (Sobel & Mayer, 1973), and as many actions of 3':5'-cyclic AMP are mediated through protein phosphorylation (Rubin & Rosen, 1975), the phosphorylation of other proteins of the excitation-contraction system will be briefly discussed. As was mentioned in the introduction, phosphorylation of cardiac sarcoplasmic reticulum *in vitro* by 3':5'-cyclic AMP-dependent protein

kinase, leading to increased Ca^{2+} uptake, has been known for some time (Kirchberger *et al.*, 1972; Tada *et al.*, 1974; Katz *et al.*, 1975). Although the significance *in vivo* of these findings is as yet unclear, it has been proposed (Tada *et al.*, 1974) that the increased Ca^{2+} uptake leads to increased contractility by abbreviation of systole. Phosphorylation of sarcolemma vesicles from skeletal and cardiac muscle also caused an increased Ca^{2+} uptake (Will *et al.*, 1973; Sulakhe & Drummond, 1974; Krause *et al.*, 1975). There are thus two potential mechanisms that could alter Ca^{2+} distribution in cardiac muscle, leading to changes in contractility.

The contractile system itself could also be modified by phosphorylation of proteins other than troponin I. Troponin T as isolated from skeletal muscle contained endogenous phosphate, and was also phosphorylated *in vitro* (Stull *et al.*, 1972; Pratje & Heilmeyer, 1972; England *et al.*, 1973; Perry & Cole, 1974). Incubation of cardiac troponin with protein kinase did not cause phosphorylation of troponin T (Cole & Perry, 1975), and the possible role of troponin T phosphorylation in the changes in contractility is uncertain. One of the light chains of myosin in skeletal and cardiac muscle has also been shown to be phosphorylated by a specific kinase (Perrie *et al.*, 1973; Pires *et al.*, 1974; Frearson & Perry, 1975). Phosphorylation of myosin light chains in platelets caused an increase in actomyosin adenosine triphosphatase activity (Adelstein & Conti, 1975), although this has not been reported for muscle. This phosphorylation represents another possible mechanism whereby contraction could be modified, although as the light-chain kinase is activated by Ca^{2+} and not by 3':5'-cyclic AMP (Pires *et al.*, 1974) modification of Ca^{2+} concentration as discussed above would first be necessary. Therefore there are several potential mechanisms whereby cardiac contractility could be modulated by protein phosphorylation in response to increased 3':5'-cyclic AMP concentration, of which troponin I phosphorylation is but one.

I thank Mrs. Susan Fielding for excellent technical assistance, Mr. Keith Ray for the gift of phosphorylase *b* and phosphorylase kinase, and Dr. Janet Albano for help with the assays for cyclic nucleotides. This work is supported by a grant from the Medical Research Council.

References

- Adelstein, R. S. & Conti, M. A. (1975) *Nature (London)* **256**, 597–598
- Bailey, C. & Villar-Palasi, C. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 1147
- Brown, B. L., Albano, J. D. M., Ekins, R. P., Sgherzi, A. M. & Tampion, W. (1971) *Biochem. J.* **121**, 561–562
- Cohen, P. (1973) *Eur. J. Biochem.* **34**, 1–14
- Cohen, P. & Antoniwi, J. F. (1973) *FEBS Lett.* **34**, 43–47
- Cohen, P., Watson, D. C. & Dixon, G. M. (1975) *Eur. J. Biochem.* **51**, 79–92
- Cole, H. A. & Perry, S. V. (1975) *Biochem. J.* **149**, 525–533
- England, P. J. (1975) *FEBS Lett.* **50**, 57–60
- England, P. J. & Walsh, D. A. (1976) *Anal. Biochem.* **75**, in the press
- England, P. J., Stull, J. T., Huang, T. S. & Krebs, E. G. (1973) *Metab. Interconvers. Enzymes* **3**, 175–184
- Farah, A. & Tuttle, R. (1960) *J. Pharmacol. Exp. Ther.* **129**, 49–55
- Fischer, E. M. & Krebs, E. G. (1958) *J. Biol. Chem.* **231**, 65–71
- Frearson, N. & Perry, S. V. (1975) *Biochem. J.* **151**, 99–107
- George, W. J., Wilkerson, R. D. & Kadowitz, P. J. (1973) *J. Pharmacol. Exp. Ther.* **184**, 228–234
- Glynn, I. M. & Chappell, J. B. (1964) *Biochem. J.* **90**, 147–149
- Goodman, L. S. & Gilman, A. (1970) *The Pharmacological Basis of Therapeutics*, 4th edn., pp. 499–501, Macmillan, New York
- Greaser, M. L. & Gergely, J. (1971) *J. Biol. Chem.* **246**, 4226–4233
- Hardman, J. G., Mayer, S. E. & Clark, B. (1965) *J. Pharmacol. Exp. Ther.* **150**, 341–348
- Huang, F. L. & Glinsman, W. H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3004–3008
- Itaya, K. & Ui, M. (1966) *Clin. Chim. Acta* **14**, 361–366
- Katz, A. M., Tada, M. & Kirchberger, M. A. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 453–472
- Kirchberger, M. A., Tada, M., Repke, D. I. & Katz, A. M. (1972) *J. Mol. Cell. Cardiol.* **4**, 673–680
- Krause, E. G., Will, H., Schirpke, B. & Wollenberger, A. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 473–490
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Kukovetz, W. R., Pock, G. & Wurm, A. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 395–414
- Lamprecht, W. & Trautschold, I. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 543–551, Verlag-Chemie, Weinheim
- Lucchesi, B. R. (1968) *Circ. Res.* **22**, 777–787
- Mayer, S. E., Namm, D. M. & Rice, L. (1970) *Circ. Res.* **26**, 225–233
- Meisler, M. H. & Langan, T. A. (1969) *J. Biol. Chem.* **244**, 4961–4968
- Nimmo, H. G. & Cohen, P. (1974) *FEBS Lett.* **47**, 162–166
- Osnes, J.-B. & Øye, I. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 415–433
- Perrie, W. T., Smillie, L. B. & Perry, S. V. (1973) *Biochem. J.* **135**, 151–164
- Perry, S. V. & Cole, H. A. (1973) *Biochem. J.* **131**, 425–428
- Perry, S. V. & Cole, H. A. (1974) *Biochem. J.* **141**, 733–743
- Pires, E., Perry, S. V. & Thomas, M. A. W. (1974) *FEBS Lett.* **41**, 292–296
- Pratje, E. & Heilmeyer, L. M. G., Jr. (1972) *FEBS Lett.* **27**, 89–93
- Ray, K. P. & England, P. J. (1976a) *Biochem. J.* **157**, 369–380
- Ray, K. P. & England, P. J. (1976b) *FEBS Lett.* in the press
- Reddy, Y. S., Ballard, D., Giri, N. Y. & Schwartz, A. (1973) *J. Mol. Cell. Cardiol.* **5**, 461–471

- Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1986-1995
- Rubin, C. S. & Rosen, O. M. (1975) *Annu. Rev. Biochem.* **44**, 831-887
- Rubio, R., Bailey, C. & Villar-Palasi, C. (1975) *J. Cyclic Nucleotide Res.* **1**, 143-150
- Sobel, B. E. & Mayer, S. E. (1973) *Circ. Res.* **32**, 407-414
- Stull, J. T. & Mayer, S. E. (1971) *J. Biol. Chem.* **246**, 5716-5723
- Stull, J. T., Brostrom, C. O. & Krebs, E. G. (1972) *J. Biol. Chem.* **247**, 5272-5274
- Sulakhe, P. V. & Drummond, G. I. (1974) *Arch. Biochem. Biophys.* **161**, 448-455
- Sutherland, E. W., Robison, G. A. & Butcher, R. W. (1968) *Circulation* **37**, 279-306
- Syska, M., Perry, S. V. & Trayer, I. P. (1974) *FEBS Lett.* **40**, 253-257
- Tada, M., Kirchberger, M. A., Repke, D. I. & Katz, A. M. (1974) *J. Biol. Chem.* **249**, 6174-6180
- Tishler, P. V. & Epstein, C. J. (1968) *Anal. Biochem.* **22**, 89-98
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Will, M., Schirpke, B. & Wollenberger, A. (1973) *Acta Biol. Med. Ger.* **31**, K45-52
- Wollenberger, A., Ristau, O. & Schoffa, G. (1960) *Pflügers Arch. Gesamte Physiol. Menschen Tieren* **270**, 399-412